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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b> <b>A61K 37/02, 39/395, 31/52</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 94/08609</b> <b>(43) International Publication Date:</b> 28 April 1994 (28.04.94)
<b>(21) International Application Number:</b> PCT/US93/09830 <b>(22) International Filing Date:</b> 14 October 1993 (14.10.93)  <b>(30) Priority data:</b> 961,792 15 October 1992 (15.10.92) US  <b>(71) Applicant:</b> DANA-FARBER CANCER INSTITUTE, INC. [US/US]; 44 Binney Street, Boston, MA 02115 (US).  <b>(72) Inventors:</b> HOTAMISLIGIL, Gökhan, S. ; 106 13th Street, #310, Charlestown, MA 02129 (US). SIEGELMAN, Bruce, M. ; 271 Waban Avenue, Waban, MA 02168 (US).  <b>(74) Agents:</b> SCHURGIN, Stanley, M. et al.; Weingarten, Schurgin, Gagnebin & Hayes, Ten Post Office Square, Boston, MA 02109 (US).		<b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> TREATMENT OF INSULIN RESISTANCE IN OBESITY LINKED TYPE II DIABETES USING ANTAGON- ISTS TO TNF- $\alpha$ FUNCTION  <b>(57) Abstract</b>  An induction of TNF- $\alpha$ mRNA expression has been observed in adipose tissue from four different insulin resistant rodent models of obesity and diabetes. TNF- $\alpha$ protein was also elevated locally and systemically. Neutralization of TNF- $\alpha$ in obese fa/fa rats caused a significant increase in the peripheral uptake of glucose in response to insulin. A method of treating an animal suffering from insulin resistance in obesity linked Type II diabetes mellitus is disclosed. The method includes providing a therapeutic agent that includes an antagonist to TNF- $\alpha$ function in a pharmaceutically acceptable carrier substance and administering a pharmacologically effective amount of the therapeutic agent to the animal.		

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TREATMENT OF INSULIN RESISTANCE IN OBESITY LINKED TYPE II  
DIABETES USING ANTAGONISTS TO TNF- $\alpha$  FUNCTION

BACKGROUND OF THE INVENTION

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Obesity and diabetes are among the most common human health problems in industrialized societies. Obesity, which is the result of an imbalance between caloric intake and energy expenditure, is highly correlated with insulin resistance and diabetes in experimental animals and humans. However, the molecular mechanisms that are involved in obesity-diabetes syndromes are not clear. Since adipose tissue is the major site for energy storage and mobilization, many investigators have focused on finding abnormalities in adipocyte physiology or metabolism (Plata-Salaman, Brain Behav. Immun. 3:193, 1989; Lardy et al., Annu. Rev. Biochem. 59:689, 1990).

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It has been shown that several cytokines such as tumor necrosis factor (TNF)- $\alpha$  have direct effects on adipocyte metabolism as well as other important metabolic actions (Le et al., Lab. Invest. 56:234, 1987; Dinarello, Immunol. Lett. 16:227, 1987; Kunkel et al., Crit. Rev. Immunol. 9:93, 1989; Grunfeld et al., Biotherapy 3:143, 1991). TNF- $\alpha$  acts in vitro on murine adipocytes to suppress expression of most adipose specific genes including enzymes involved in lipogenesis (Kawakami et al., Proc. Natl. Acad. Sci. USA 79:912, 1982; Price et al., Arch. Biochem. Biophys. 251:738, 1986). However, some of these effects are not observed in primary cultures of human or rat adipocytes (Grunfeld et al., Biotherapy 3:143, 1991; Kern, J. Lipid Res. 29:909, 1988).

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In vivo, TNF- $\alpha$  expression has been associated with catabolic states leading to a "wasting syndrome," termed cachexia (Beutler et al., Nature 316:552, 1985; Beutler et al., Science 232:977, 1986; Beutler et al., Nature 320:584, 1986; Oliff et al., Cell 50:555, 1987; Beutler et al., Ann. Rev. Immunol. 7:625, 1989), but this effect of TNF- $\alpha$  has been

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challenged by several groups of investigators (Semb et al., J. Biol. Chem. 262:8390, 1987; Grunfeld et al., J. Lipid Res. 30:579, 1989; Feingold et al., J. Clin. Invest. 83:1116, 1989; Patton et al., J. Clin. Invest. 80:1587 (1987); Kettlehut et al., J. Clin. Invest. 81:1384, 1988; Tracey et al., J. Clin. Invest. 86:2014, 1990; Socher et al., J. Exp. Med. 167:1957, 1988; Mullen et al., Proc. Soc. Exp. Biol. Med. 193:318, 1990; Teng et al., Proc. Natl. Acad. Sci. USA 88:3535, 1991; for reviews see C. Grunfeld et al., Cancer Res. 49:2554, 1989; Fiers, FEBS 285:199, 1991).

TNF- $\alpha$  administration causes an increase in serum triglycerides and very low density lipoproteins in rats and humans (Semb et al., J. Biol. Chem. 262:8390, 1987; Grunfeld et al., J. Lipid Res. 30:579, 1989; Feingold et al., J. Clin. Invest. 83:1116, 1989; Sherman et al., J. Clin. Oncol. 6:344, 1988). This hyperlipidemia is thought to be the result of decreased lipoprotein lipase activity and increased hepatic lipogenesis (Feingold et al., J. Clin. Invest. 80:184, 1987). TNF- $\alpha$  administration also has effects on appetite and gastrointestinal tract functions (Plata-Salaman, Brain Behav. Immun. 3:193, 1989). Besides TNF- $\alpha$ , other cytokines such as TNF- $\beta$ , IL-1, IL-6 and interferon (INF) also have profound effects on lipid metabolism (Grunfeld et al., Biotherapy 3:143, 1991). Furthermore, all of these cytokines affect glucose homeostasis in various tissues (Rey et al., Am. J. Physiol. 253:R794, 1987; Meszaros et al., Biochem. Biophys. Res. Comm. 149:1, 1987; Koivisto et al., Diabetes 38:641, 1989; Snick, Annu. Rev. Immunol. 8:253, 1990).

Previous studies have also suggested an association of TNF- $\alpha$  with states of peripheral insulin resistance, especially in infection. First, it is established that biological mediator(s) generated during infection interfere with insulin's actions and lead to profound metabolic alterations (Beutler et al., Ann. Rev. Immunol. 7:625, 1989; Stephens et al., J. Biol. Chem. 266:21839, 1991; Beisel, Ann. Rev. Med. 26:9, 1975; Stephens et al., Biochem. Biophys. Res

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Common. 183:417, 1992). Second, incorporation of glucose into lipids is decreased upon short term treatment of 3T3-L1 cells with supernatants of activated macrophages (Olney, Science 164:719, 1969; Cameron et al., Clin. Exp. Pharmacol. Physiol. 5:41, 1978), and third, treatment of L6 myotubes (Cornelius et al., J. Biol. Chem. 265:20506, 1990) and 3T3-L1 adipocytes with recombinant TNF- $\alpha$  causes downregulation of Glut4 expression (Stephens et al., J. Biol. Chem. 266:21839, 1991). However, the specificity of TNF- $\alpha$ 's effect on Glut4 mRNA in fat cells was not clear in that expression of many or most other fat cell genes was also affected (Stephens et al., J. Biol. Chem. 266:21839, 1991). Finally, a recent study has directly demonstrated that chronic, low level administration of TNF- $\alpha$  to rodents induces systemic insulin resistance (Lang et al., Endocrinology 130:43, 1992).

Insulin resistance, defined as a smaller than expected biological response to a given dose of insulin, is a ubiquitous correlate of obesity. Indeed, many of the pathological consequences of obesity are thought to involve insulin resistance. These include hypertension, hyperlipidemia and, most notably, non-insulin dependent diabetes mellitus (NIDDM). Most NIDDM patients are obese, and a very central and early component in the development of NIDDM is insulin resistance (reviewed in Moller et al., New Eng. J. Med. 325:938, 1991). It has been demonstrated that a post-receptor abnormality develops during the course of insulin resistance, in addition to the insulin receptor downregulation during the initial phases of this disease (Olefsky et al., in Diabetes Mellitus, H. Rifkin and D. Porte, Jr., Eds. (Elsevier Science Publishing Co., Inc., New York, ed. 4, 1990), pp. 121-153). Several studies on glucose transport systems as potential sites for such a post-receptor defect have demonstrated that both the quantity and function of the insulin sensitive glucose transporter (Glut4) is deficient in insulin resistant states of rodents and humans (Garvey et al., Science 245:60, 1989; Sivitz et al., Nature

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340:72, 1989; Berger et al., Nature 340:70, 1989; Kahn et al., J. Clin. Invest. 84:404, 1989; Charron et al., J. Biol. Chem. 265:7994, 1990; Dohm et al., Am. J. Physiol. 260:E459, 1991; Sinha et al., Diabetes 40:472, 1991; Friedman et al., J. Clin. Invest. 89:701, 1992). A lack of a normal pool of insulin-sensitive glucose transporters could theoretically render an individual insulin resistant (Olefsky et al., in Diabetes Mellitus, H. Rifkin and D. Porte, Jr., Eds. (Elsevier Science Publishing Co., Inc., New York, ed. 4, 1990), pp. 121-153). However, some studies have failed to show downregulation of Glut4 in human NIDDM, especially in muscle, the major site of glucose disposal (for a review see G.I. Bell, Diabetes 40:413, 1990; Pederson et al., Diabetes 39:865, 1990; Handberg et al., Diabetologia 33:625, 1990; Garvey et al., Diabetes 41:465, 1992).

The mechanistic link between obesity and insulin resistance is not understood. Much attention has been focused on the role of free fatty acids as potential mediators of insulin resistance (Reaven et al., Am. J. Med. 85:106, 1988; Lonroth, J. Intern. Med. Suppl. 735:23, 1991; Bjorntorp, Diabetes Care 14:1132, 1991). Free fatty acid levels are typically elevated in obesity, and fatty acids have been shown to affect insulin sensitivity in vitro and in vivo (Reaven et al., Am. J. Med. 85:106, 1988; Lonroth, J. Intern. Med. Suppl. 735:23, 1991; Bjorntorp, Diabetes Care 14:1132, 1991).

#### SUMMARY OF THE INVENTION

It has surprisingly been shown that an induction of TNF- $\alpha$  mRNA expression can be observed in adipose tissue from four different insulin resistant rodent models of obesity and diabetes. TNF- $\alpha$  protein is also elevated locally and systemically. Neutralization of TNF- $\alpha$  in obese fa/fa rats with an antagonist to TNF- $\alpha$  function causes a significant increase in the peripheral uptake of glucose in response to insulin, thus overcoming inherent insulin resistance.

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Thus, the invention generally features a method of treating an animal suffering from insulin resistance in obesity linked Type II diabetes mellitus. The method includes providing a therapeutic agent that includes an antagonist to TNF- $\alpha$  function and a pharmaceutically acceptable carrier substance and administering to the animal a pharmacologically effective amount of the therapeutic agent.

Preferably, the therapeutic agent includes as antagonist to TNF- $\alpha$  function a receptor, most preferably a TNF- $\alpha$  receptor or effective portion thereof; a monoclonal antibody, most preferably an anti-TNF- $\alpha$  monoclonal antibody or effective portion thereof; or an agent capable of suppressing production of TNF- $\alpha$  or of TNF- $\alpha$  mRNA, most preferably pentoxifylline. Additionally, the agent preferably includes immunoglobulin, most preferably in a chimeric complex with the antagonist to TNF- $\alpha$  function.

As used herein the term "antagonist to TNF- $\alpha$  function" includes any agent that interacts with TNF- $\alpha$  and interferes with its function, e.g., antibody or portions thereof reactive with TNF- $\alpha$ , the TNF- $\alpha$  receptor or portions thereof reactive with TNF- $\alpha$ , or any other ligand which binds to TNF- $\alpha$ . The term also includes any agent that will interfere in the overproduction of TNF- $\alpha$  mRNA or TNF- $\alpha$  protein or antagonize one or both TNF- $\alpha$  receptors. Such agents may be in the form of chimeric hybrids, useful for combining the function of the agent with a carrier protein to increase the serum half-life of the therapeutic agent or to confer cross-species tolerance.

Other features and advantages of the invention will be found in the following description of the preferred embodiments thereof and in the claims.

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DESCRIPTION OF THE PREFERRED EMBODIMENTS

The discovery that TNF- $\alpha$  expression is elevated in rodent models of obesity and diabetes has permitted the development of a therapeutic treatment for overcoming the insulin resistance associated with obesity linked Type II diabetes mellitus. Described below are experiments that led to this discovery.

To examine the expression of the TNF- $\alpha$  gene in the tissues of lean (+/?) or obese (db/db) mice, total RNA was extracted from various tissues and organs and subjected to RNA (Northern blot) analysis (Fig. 1). Endogenous expression was evident only in adipose tissue and spleen. The level of TNF- $\alpha$  mRNA expression in spleen was not different in obese mice compared to their lean litter mates. However, in adipose tissue the amount of TNF- $\alpha$  mRNA per unit of RNA was at least 5-10 fold elevated in obese animals compared to lean controls. TNF- $\beta$ , IL-1- $\alpha$  and - $\beta$  and IL-6 were neither expressed in fat tissue nor regulated in obesity in any other organ. The earliest time of adipose expression of TNF- $\alpha$  examined was 6-7 weeks of age in db/db mice and 3-4 weeks of age in fa/fa rats, when animals are known to be obese and insulin resistant, but not significantly hyperglycemic (Coleman, Diabetes 31:1, 1982; Shafrir, in Diabetes Mellitus; H. Rifkin and D. Porte, Jr. Eds. (Elsevier Science Publishing Co., Inc., New York, ed. 4, 1990), pp. 299-340). TNF- $\alpha$  mRNA in fat tissue was elevated at these times.

Adipose tissue consists of vascular endothelial cells, smooth muscle cells, fibroblasts, local mast cells and macrophages besides adipocytes (Wasserman, in Handbook of Physiology, A.E. Renold and G.F. Cahill, Eds. (Am. Physiol. Soc., Washington D.C., 1965), vol. 5, pp. 87-100). To determine the source of TNF- $\alpha$  expression in adipose tissue, mature adipocytes and non-adipose cells (stromal-vascular fraction) were separated as described (Rodbell, J. Biol. Chem. 239:375, 1964), and the amount of mRNA associated with these compartments was determined. The majority of the TNF- $\alpha$



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mRNA fractionated with the adipocytes although some was also detected in the stromal-vascular fraction that contains non-adipocytes plus less mature adipocytes (Fig. 2). These results suggest that adipocytes express TNF- $\alpha$  mRNA *in vivo* and are the major source of the elevated levels of mRNA expression in adipose tissue.

As metabolic profiles differ among various animal models of obesity and diabetes, analysis of multiple models was undertaken to separate the effects of hyperinsulinemia, hyperglycemia and obesity. The diabetes (db/db) and obese (ob/ob) mice are characterized by massive obesity, hyperphagia, variable hyperglycemia, insulin resistance, hyperinsulinemia and impaired thermogenesis (Coleman, *Diabetes* 31:1, 1982; E. Shafrir, in *Diabetes Mellitus*; H. Rifkin and D. Porte, Jr. Eds. (Elsevier Science Publishing Co., Inc., New York, ed. 4, 1990), pp. 299-340). However, diabetes is much more severe in the db/db model (Coleman, *Diabetes* 31:1, 1982; E. Shafrir, in *Diabetes Mellitus*; H. Rifkin and D. Porte, Jr. Eds. (Elsevier Science Publishing Co., Inc., New York, ed. 4, 1990), pp. 299-340). Zucker (fa/fa) rats are severely obese, hyperinsulinemic, and insulin resistant (Coleman, *Diabetes* 31:1, 1982; E. Shafrir, in *Diabetes Mellitus*; H. Rifkin and D. Porte, Jr. Eds. (Elsevier Science Publishing Co., Inc., New York, ed. 4, 1990), pp. 299-340), and the fa/fa mutation may be the rat equivalent of the murine db mutation (Friedman et al., *Cell* 69:217-220, 1992; Truett et al., *Proc. Natl. Acad. Sci. USA* 88:7806, 1991). Tubby (tub/tub) mice are characterized by obesity, moderate insulin resistance and hyperinsulinemia without significant hyperglycemia (Coleman et al., *J. Heredity* 81:424, 1990). Like the db/db mouse, the ob/ob, tub/tub and fa/fa models exhibit a similar obesity related expression of TNF- $\alpha$  mRNA in fat (Fig. 3).

The monosodium glutamate (MSG) model for chemically-induced obesity (Olney, *Science* 164:719, 1969; Cameron et al., *Cli. Exp. Pharmacol. Physiol.* 5:41, 1978), in which

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obesity is less severe than in the genetic models and develops without hyperphagia, hyperinsulinemia and insulin resistance, was also examined. No induction of TNF- $\alpha$  mRNA in MSG-treated animals was observed (Fig. 3). Finally, the streptozotocin (STZ) model for chemically-induced diabetes was tested to examine the effects of hyperglycemia in the absence of obesity. STZ-treated animals are deficient in insulin and severely hyperglycemic (Coleman, Diabetes 31:1, 1982; E. Shafrir, in Diabetes Mellitus; H. Rifkin and D. Porte, Jr. Eds. (Elsevier Science Publishing Co., Inc., New York, ed. 4, 1990), pp. 299-340). STZ-treated rats did not exhibit induction of TNF- $\alpha$  expression in fat tissue. These results suggest that TNF- $\alpha$  induction is best correlated with severe obesity and insulin resistance. Detection of elevated TNF- $\alpha$  gene expression in four independent animal models suggests that this may be a general phenomenon in these disorders.

The differences in mRNA levels in the adipose tissues of lean and obese animals are also reflected in the amounts of local and systemic (circulating) TNF- $\alpha$  protein. Local protein production was examined in explanted adipose tissue, and a significant amount of TNF- $\alpha$  secretion was observed. When expressed as the mass of TNF- $\alpha$  secreted per unit of tissue DNA, the obese adipose tissue secreted approximately twice as much TNF- $\alpha$  as the lean tissue. The levels of TNF- $\alpha$  in circulation were determined by the ELISA assay in plasma of 24 control and db/db animals. Only 6/24 (25%) lean animals had detectable levels of TNF- $\alpha$  protein, with levels ranging from 25 to 97.7 pg/ml ( $61.53 \pm 11.9$ ). In obese animals TNF- $\alpha$  protein was detectable in 14/24 (58.3%), with levels ranging from 34 to 165.6 pg/ml ( $85.6 \pm 10.0$ ). These differences in the fraction of lean or obese animals having detectable TNF- $\alpha$  levels in the plasma were statistically significant with a p value  $< 0.05$ , indicating that TNF- $\alpha$  protein in circulation is also elevated in obese animals.

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However, the circulating protein concentrations detected in plasma were quite low.

Most studies examining the effects of TNF- $\alpha$  on adipocytes have reported a general suppression of fat cell gene expression and, in some cases, a dedifferentiation response (Torti et al., Science 229:867, 1985; Pekala et al., J. Exp. Med. 157:1360, 1983; Ron et al., J. Clin. Invest. 89:223, 1992; Cornelius et al., J. Biol. Chem. 265:20506, 1990; Stephens et al., J. Biol. Chem. 266:21839, 1991).

However, the interpretation of most of these studies is complicated by the fact that very high doses of mixed cytokines or human TNF- $\alpha$  were often used, and it is now known that recombinant human TNF- $\alpha$  binds only to one of the two murine TNF receptors (Lewis et al., Proc. Natl. Acad. Sci. USA 88:2830, 1991). Because of the results presented above,

we have examined the chronic effects (10-15 days) of low dose (50pM; 2ng/ml) murine TNF- $\alpha$  treatment on cultured murine fat cells. This treatment did not cause any phenotypic changes in 3T3-F442A adipocytes. We then examined the pattern of specific mRNA, especially that for adipsin and Glut4, the insulin-sensitive glucose transporter that is expressed in muscle and fat. Both of these genes are expressed in a differentiation-dependent manner in adipocytes and are specifically downregulated in obesity-insulin resistance syndromes (Flier et al., Science 237:405, 1987; Rosen et al., Science 244:1483, 1989; Choy et al., J. Biol. Chem. 267:12736-12741, 1992; Garvey et al., Science 245:60, 1989; Sivitz et al., Nature 340:72, 1989; Berger et al., Nature 340:70, 1989; Kahn et al., J. Clin. Invest. 84:404, 1989; Charron et al., J. Biol. Chem. 265:7994, 1990; Dohm et al., Am. J. Physiol. 260:E459, 1991; Sinha et al., Diabetes 40:472, 1991; Friedman et al., J. Clin. Invest. 89:701, 1992).

Long term treatment of adipocytes with TNF- $\alpha$  led to downregulation of Glut4 mRNA (Fig. 4a). This down regulation is not general for most fat specific genes as no changes were observed in the mRNA levels for the fatty acid binding

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protein aP2, and glycerophosphate dehydrogenase (GPD). Glut1 and  $\beta$ -actin mRNA were also unaffected. However, a dramatic reduction in adipsin mRNA was evident (Fig. 4a). The gene expression pattern of these cells is strikingly similar to that of adipose tissue in obese animals (Fig. 4b) where Glut4 and adipsin mRNA expression are also severely deficient but most other fat specific genes are expressed fairly normally (Flier et al., Science 237:405, 1987; Rosen et al., Science 244:1483, 1989; Choy et al., J. Biol. Chem. 267:12736-12741, 1992). These results strongly suggest that TNF- $\alpha$  could be a key mediator of abnormal gene expression in obesity-diabetes syndromes and may affect glucose homeostasis.

To use the results of the above studies in developing a treatment regimen for insulin resistance, a neutralization of TNF- $\alpha$  *in vivo* was undertaken and its effect on glucose homeostasis of genetically obese and insulin resistant animals examined. For neutralization, a recombinant soluble TNF- $\alpha$  receptor-IgG chimeric protein (TNFR-IgG, Genentech, Inc., San Francisco, CA) was used (Ashkenazi et al., Proc. Natl. Acad. Sci. USA 88:10535, 1992). This molecule was administered *iv* into fa/fa rats daily, for 3 days (200  $\mu$ g/rat), and a steady blood level of  $47.69 \pm 4.79$  ng/ml was established (Ashkenazi et al., Proc. Natl. Acad. Sci. USA 88:10535, 1992; plasma TNFR-IgG assays were done by ELISA (Bender Medsystems, Vienna, Austria). *In vivo* insulin sensitivity was then examined by utilizing two-step hyperinsulinemic-euglycemic clamps according to the following protocol.

Upon arrival, Zucker obese rats (fa/fa) were housed for at least a week prior to experimental procedures. Surgeries for the placement of jugular vein and carotid artery catheters were performed under sterile conditions using ketamine and xylazine (*i.m.*) anesthesia. After surgery, all rats were allowed to regain consciousness and placed in individual cages. TNFR-IgG (200  $\mu$ g/rat in 200  $\mu$ l volume) or vehicle (20% glycerol in PBS; 200  $\mu$ l/rat) was administered

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through the jugular vein after complete recovery and for the following two days. Sixteen hours after the last treatment, hyperinsulinemic-euglycemic clamps were performed. Rats were placed in restrainers and a bolus of 4  $\mu$ Ci [ $3\text{-}^3\text{H}$ ] glucose (NEN) was administered, followed by a continuous infusion of the tracer at a dose of 0.2  $\mu$ Ci/min (20  $\mu$ l/min). Two hours after the start of the tracer infusion, 3 blood samples (0.3 ml each) were collected at 10 minute intervals (-20-0 min) for basal measurements. An insulin infusion was then started (5 mU/kg/min), and 100  $\mu$ l blood samples were taken every 10 min. to monitor plasma glucose. A 30% glucose solution was infused using a second pump based on the plasma glucose levels in order to reach and maintain euglycemia. Once a steady state was established at 5 mU/kg/min insulin (stable glucose infusion rate and plasma glucose), 3 additional blood samples (0.3 ml each) were obtained for measurements of glucose, [ $3\text{-}^3\text{H}$ ] glucose and insulin (100-120 min.). A higher dose of insulin (25 mU/kg/min.) was then administered and glucose infusion rates were adjusted for the second euglycemic clamp and blood samples were taken at min. 220-240. Glucose specific activity was determined in deproteinized plasma and the calculations of Rd and HGO were made, as described (Lang et al., Endocrinology 130:43, 1992). Plasma insulin levels at basal period and after 5 and 25 mU/kg/min. infusions were  $102.6 \pm 9.4$ ,  $188.4 \pm 41.4$  and  $667.4 \pm 76.0$  ng/ml in controls and  $95.46 \pm 12.4$ ,  $200.5 \pm 23.6$  and  $659.1 \pm 39.7$  ng/ml in TNFR-IgG-treated animals.

In summary, plasma insulin levels following 2 doses of constant insulin infusion (5 mU and 25 mU/kg/min) were similar in control and TNFR-IgG-treated animals. Plasma glucose levels (Fig. 5a) and glucose infusion rates to maintain euglycemia under hyperinsulinemia (Fig. 5b) were stable in both groups of animals during the clamps. However, at all time points and at the two different insulin doses, TNFR-IgG-treated animals required 2-3 times more glucose to

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maintain normal blood glucose levels, indicating a greater response to insulin.

Insulin regulation of glucose homeostasis has two major components; stimulation of peripheral glucose uptake and suppression of hepatic glucose output. Using tracer studies in the glucose clamps, it was possible to determine which portion of the insulin response was affected by the soluble receptor. As illustrated in Fig. 6a, insulin-stimulated peripheral glucose utilization rate ( $R_d$ ) was 2-3 fold higher in the TNFR-IgG-treated animals while hepatic glucose output (HGO) was unaffected (Fig. 6b). Thus, the neutralization of  $TNF-\alpha$  has profound effects on the sensitivity to insulin in obese-diabetic animals. This effect is predominantly seen as increased peripheral glucose uptake.

Not being bound by any theory, one must still address the question of how a putative role for  $TNF-\alpha$  in obesity-linked insulin resistance can be reconciled with its possible role in cachexia (Beutler et al., Nature 316:552, 1985, Beutler et al., Science 232:997, 1986; Beutler et al., Nature 320:584, 1986, Oliff et al., Cell 50:555, 1987). Clearly, this appears to be a question of the hormonal milieu of the organism and the relative levels of this cytokine (Fraker et al., Am. J. Physiol. 256:E725, 1989). The levels produced in the obese rodents or those that yield insulin resistance when given exogenously (Lang et al., Endocrinology 130:43, 1992) are far lower than those which can induce a variety of other symptoms, including cachexia (Beutler et al., Nature 316:552, 1985; Beutler et al., A. Cerami., Science 232:977, 1986; Beutler et al., Nature 320:584, 1986; Oliff et al., Cell 50:555, 1987). These dose dependent differences in biological effects are especially in accord with recent data demonstrating at least two different receptor systems, having different affinities for  $TNF-\alpha$  (Lewis et al., Proc. Natl. Acad. Sci. USA 88:2830, 1991; Goodwin et al., Mol. Cell Biol. 11:3020, 1991; Tartaglia et al., Proc. Natl. Acad. Sci. USA 88:9292, 1991).

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Materials and Methods

**Expression of TNF- $\alpha$  mRNA in the tissues of lean and obese mice.** Total RNA from tissues of 7-8 week old, male, lean (+/?) and obese (db/db) animals (Jackson Laboratories, Bar Harbor, ME), were extracted by a cesium chloride extraction protocol (Chirgwin et al., Biochemistry 18:5294, 1979). Total RNA (20  $\mu$ g) was denatured in formamide and formaldehyde at 55°C for 15 min. and separated by electrophoresis in formaldehyde-containing agarose gels, as described (Maniatis et al., Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, ed. 2, 1989). RNA was blotted onto Biotrans membranes, UV-crosslinked (Stratagene) and baked for 0.5 hours. Hybridization and washes were done as directed by the manufacturer. DNA probes were radioactively labeled to specific activities of at least  $10^9$  d.p.m./ $\mu$ g with [ $^{32}$ P]- $\alpha$ -dCTP (6000 Ci/mmol) by the random priming method (Maniatis et al., Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, ed. 2, 1989). Referring to Fig. 1, lanes 1 and 2 show epididymal fat, lanes 3 and 4 show liver, lanes 5 and 6 show kidney, lanes 7 and 8 show skeletal muscle, and lanes 9 and 10 show spleen. Odd numbered lanes show lean mice and even numbered lanes show obese.  $\beta$ -actin mRNA is shown as a control for loading and integrity of the RNA. Lean mice are designated as +/- since +/+ and db/+ animals have not been differentiated.

**TNF- $\alpha$  mRNA expression in cell-fractionated adipose tissue.**

Epididymal fat pads were isolated from 12-13 week old, male lean (+/?) and obese (db/db) mice, washed in sterile PBS, minced, washed with Krebs-Ringer bicarbonate (KRB) buffer (pH 7.4) containing 4% albumin and 5 mM glucose, and treated with collagenase (0.5 mg/ml) on a shaking platform at 37°C for 30 min. (Rodbell, J. Biol. Chem. 239:375, 1964). The incubation medium was filtered through Nitex screen filters (250  $\mu$ m pore size) to remove undigested tissue.

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Adipocytes were then separated by their ability to float upon low speed centrifugation. To obtain total stromal-vascular fractions, the medium below the adipocyte layer was centrifuged at 200X g for 10 minutes and the pellets were washed 3X with warm KRB buffer. Total RNA was extracted from fractions as described above. Referring to Fig. 2, lanes 1 and 2 show adipocyte fraction, and lanes 3 and 4 show stromal-vascular fraction. Odd numbered lanes show lean mice and even numbered lanes show obese.  $\beta$ -actin mRNA is shown as a control for loading and integrity of RNA.

Adipose expression of TNF- $\alpha$  mRNA in different rodent models of genetic and chemically induced obesity or diabetes.

Total RNA (20  $\mu$ g) from epididymal fat pads of different animal models was determined. The ob/ob, db/db and tub/tub obese mice and their lean controls were obtained from Jackson Laboratories (Bar Harbor, ME), and Zucker rat tissues were from Drs. F. Gregoire and M.R.C. Greenwood (University of California at Davis). For monosodium glutamate (MSG) treatment, 3 mg per gram body weight MSG was subcutaneously injected into neonatal mice and tissues were collected 7 weeks later. These mice were a gift from Dr. S. Ross (University of Illinois Medical School). Streptozotocin-treated rats (0.1 mg per g body weight streptozotocin (STZ) was intraperitoneally injected into 5-6 week old rats and tissues were collected 8 days later) were from Dr. R.C. Kahn (Joslin Diabetes Center, Boston, MA). The cDNA clone for murine TNF- $\alpha$ , a gift of Dr. Bruce Beutier (University of Texas, Southwestern Medical Center), was subjected to Northern blot analysis as described above. Referring to Fig. 3, lane 1 shows +/- lean mice, lane 2 shows ob/ob obese mice, lane 3 shows +/- lean mice, lane 4 shows db/db obese mice, lane 5 shows +/- lean rat, lane 6 shows fa/fa obese rat, lane 7 shows tub/+ lean mice, lane 8 shows tub/tub obese mice, lane 9 shows Swiss-Webster lean mice, lane 10 shows MSG-treated Swiss-Webster obese mice, lane 11 shows Wistar



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non-diabetic rat, and lane 12 shows STZ-treated Wistar diabetic rat. All animals were males; +/?, ob/ob, +/?, db/db, tub/+ and tub/tub mice were 12-13 weeks old; and +/? and fa/fa rats were 7-8 weeks old.  $\beta$ -actin mRNA is shown as a control for loading and integrity of the RNA.

**Effect of chronic treatment of 3T3-F442A cells with murine TNF- $\alpha$  on adipocyte gene expression.**

Referring to Fig. 4a, murine 3T3-F442A adipocytes were cultured and differentiated in 10% fetal calf serum and 5  $\mu$ g/ml insulin, as described (Dobson et al., J. Biol. Chem. 262:1804, 1987; Choy et al., J. Biol. Chem. 267:12736-12741, 1992). Adipocyte conversion was essentially complete by seven days post-confluence. Day 0 represents the time when cells were fully differentiated. Adipocytes were then treated with 50 pM recombinant murine TNF- $\alpha$  (Genzyme Corporation, Cambridge, MA) for 10 days in the same medium. Total RNA was extracted from adipocytes, as described (Dobson et al., J. Biol. Chem. 262:1804, 1987; Choy et al., J. Biol. Chem. 267:12736-12741, 1992) and subjected to Northern blot analysis and probed with cDNAs for glucose transporter type 1 (Glut1), glucose transporter type 4 (Glut4), fatty acid binding protein aP2, adipsin/complement factor D, glycerophosphate dehydrogenase (GPD) and  $\beta$ -actin. Referring to Fig. 4b, total RNA (20  $\mu$ g) from epididymal fat pads of 12-13 week old, male, +/? lean (L), and db/db obese (O) animals were subjected to Northern blot analysis and probed with the same markers as described above.

**Glucose control during hyperinsulinemic-euglycemic clamps.**

Fig. 5a shows plasma glucose levels, and Fig. 5b shows glucose infusion rates. Male, 7-9 weeks old, fa/fa rats (Charles River Laboratories, MA) were intravenously treated with 200  $\mu$ g/rat TNFR-IgG (n=8) or vehicle (20% glycerol in PBS) (n=5) for 3 consecutive days, and  $\approx$ 16 hours after the last treatment, glucose clamps were performed on conscious

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animals (34). The values represent the mean  $\pm$ SE of plasma glucose and glucose infusion rates of all animals within each group at a given time point.

**Effect of TNFR-IgG infusion on glucose homeostasis in fa/fa rats.**

Peripheral glucose utilization (Rd) and hepatic glucose uptake (HGO) were calculated, as described ((34); Beisel, Ann. Rev. Med. 26:9, 1975; Stephens et al., Biochem. Bioph. Res. Comm. 183:417, 1992). Rd increased 45.65 and 78.26% over basal upon 5 and 25 mU/kg/min insulin infusion, respectively, in TNFR-IgG-treated animals. The same doses of insulin infusions resulted in 13.84 and 31.02% increase over basal in controls. Fig. 6a shows peripheral glucose utilization, and Fig. 6b shows hepatic glucose output. The values represent the mean  $\pm$ SE of Rd and HGO of all animals in each group.

Use

The TNF- $\alpha$  receptor, anti-TNF- $\alpha$  monoclonal antibodies, or individual portions thereof can be used for therapeutic treatment to interfere with TNF- $\alpha$  binding either at the ligand or receptor level and reduce insulin resistance. In addition, any of the specific antagonists can be joined to a carrier protein to increase the serum half-life of the therapeutic agent. For example, a soluble immunoglobulin chimera such as described herein, can be obtained for each specific TNF- $\alpha$  antagonist or antagonistic portion thereof, as described in Capon et al., U.S. Patent No. 5,116,964, the whole of which is hereby incorporated by reference herein. The immunoglobulin chimera are easily purified through IgG-binding protein A-Sepharose chromatography. The chimera have the ability to form an immunoglobulin-like dimer with the concomitant higher avidity and serum half-life.

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Additionally, the therapeutic agent may be a molecule capable of suppressing production of TNF- $\alpha$  or of TNF- $\alpha$  mRNA. As shown by Strieter et al. in "Cellular and Molecular Regulation of Tumor Necrosis Factor-Alpha Production by Pentoxifylline" (Biochem. Biophys. Res. Commun. 155:1230, 1988, the whole of which is hereby incorporated by reference herein), pentoxifylline is able to suppress the production of both biologically active TNF- $\alpha$  and TNF- $\alpha$  mRNA expression by more than 50%.

A candidate antagonist can be assayed for effectiveness, e.g., via the hyperinsulinemic-euglycemic clamp technique as described herein. Alternatively, the effect of the candidate agent on reducing circulating levels of TNF- $\alpha$  can be measured in an ELISA assay. Agents believed to function by interacting with one or both TNF- $\alpha$  receptors can be examined for their effect on fat cell gene expression as described herein.

The therapeutic agents may be administered orally, topically, or parenterally, (e.g., intranasally, subcutaneously, intramuscularly, intravenously, or intra-arterially) by routine methods in pharmaceutically acceptable inert carrier substances. Optimal dosage and modes of administration can readily be determined by conventional protocols. Preferably, administration would be systemic and a decrease in insulin resistance would be manifested in a drop in circulating levels of glucose and/or insulin in the patient.

While the present invention has been described in conjunction with a preferred embodiment, one of ordinary skill, after reading the foregoing specification, will be able to effect various changes, substitutions of equivalents, and other alterations to the compositions and methods set forth herein. It is therefore intended that the protection granted by Letters Patent hereon be limited only by the definitions contained in the appended claims and equivalents thereof.

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What is claimed is:

1. A method of treating an animal suffering from insulin resistance in obesity linked Type II diabetes mellitus comprising

5 providing an animal suffering from insulin resistance in obesity linked Type II diabetes mellitus;

providing a therapeutic agent, said agent comprising an antagonist to TNF- $\alpha$  function in a pharmaceutically acceptable carrier substance, and

10 administering to said animal a pharmacologically effective amount of said therapeutic agent.

2. The method of claim 1 wherein said therapeutic agent comprises a receptor.

15 3. The method of claim 1 wherein said therapeutic agent comprises a TNF- $\alpha$  receptor or reactive portion thereof.

4. The method of claim 1 wherein said therapeutic agent comprises an antibody.

5. The method of claim 1 wherein said therapeutic agent comprises a monoclonal antibody.

20 6. The method of claim 1 wherein said therapeutic agent comprises an anti-TNF- $\alpha$  monoclonal antibody or reactive portion thereof.

7. The method of claim 1 wherein said therapeutic agent comprises an immunoglobulin.

25 8. The method of claim 1 wherein said therapeutic agent comprises immunoglobulin G.

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9. The method of claim 1 wherein said therapeutic agent comprises a protein-immunoglobulin chimeric protein.

10. The method of claim 1 wherein said therapeutic agent comprises a receptor-immunoglobulin chimeric protein.

5 11. The method of claim 1 wherein said therapeutic agent comprises a TNF- $\alpha$  receptor-immunoglobulin G chimeric protein.

12. The method of claim 1 wherein said therapeutic agent comprises a reactive portion of a TNF- $\alpha$  receptor complexed with immunoglobulin G.

10 13. The method of claim 1 wherein said therapeutic agent comprises a recombinant soluble TNF- $\alpha$  receptor-immunoglobulin G chimeric protein.

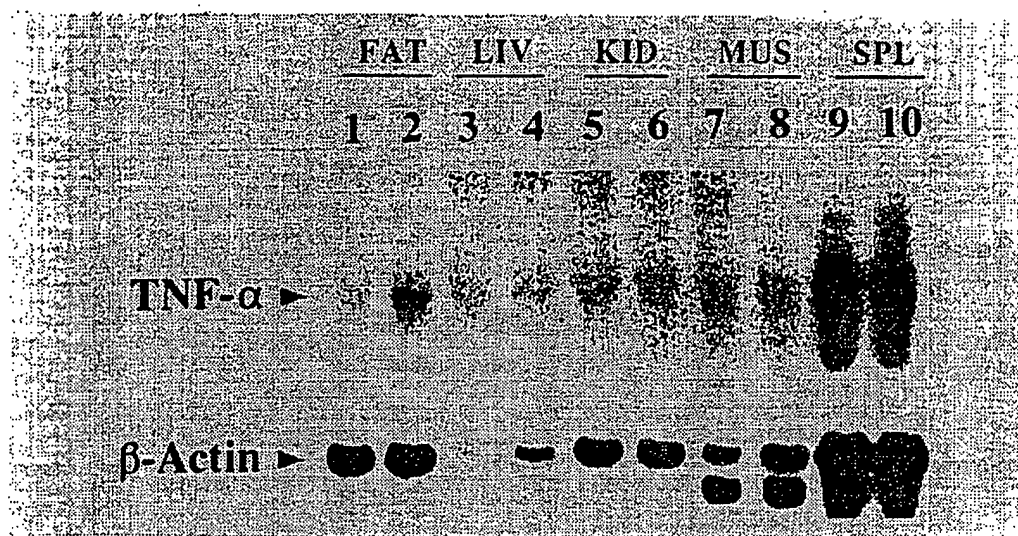
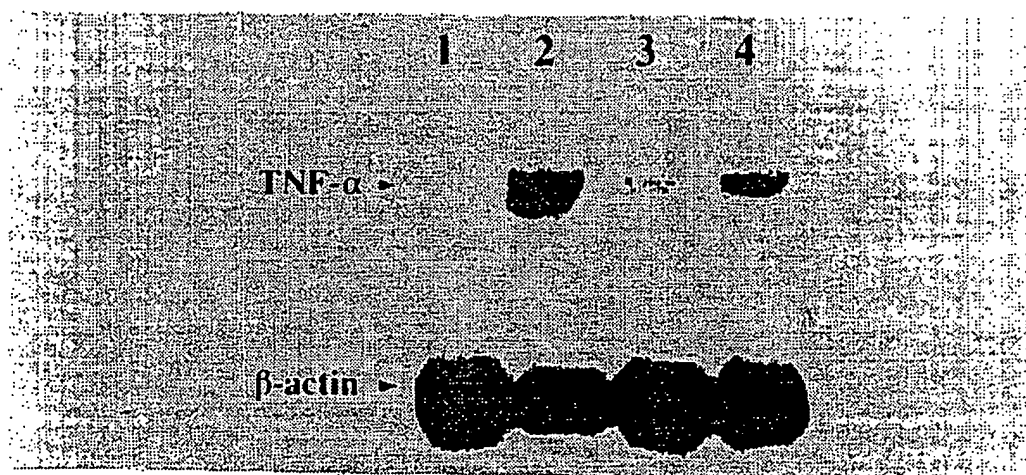
14. The method of claim 1 wherein said therapeutic agent comprises a TNF- $\alpha$  receptor antagonist.

15 15. The method of claim 1 wherein said therapeutic agent comprises a reactive portion of a TNF- $\alpha$  receptor antagonist.

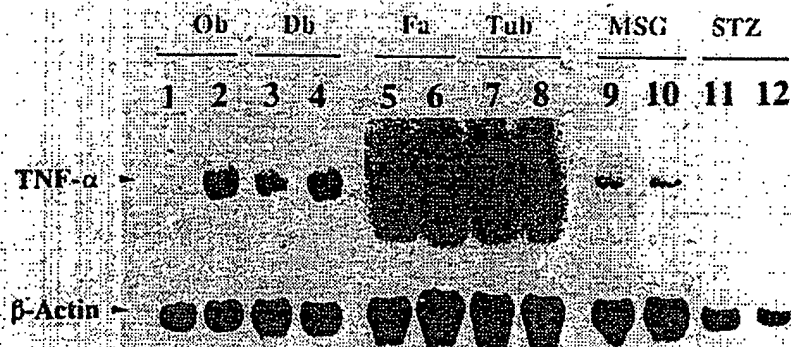
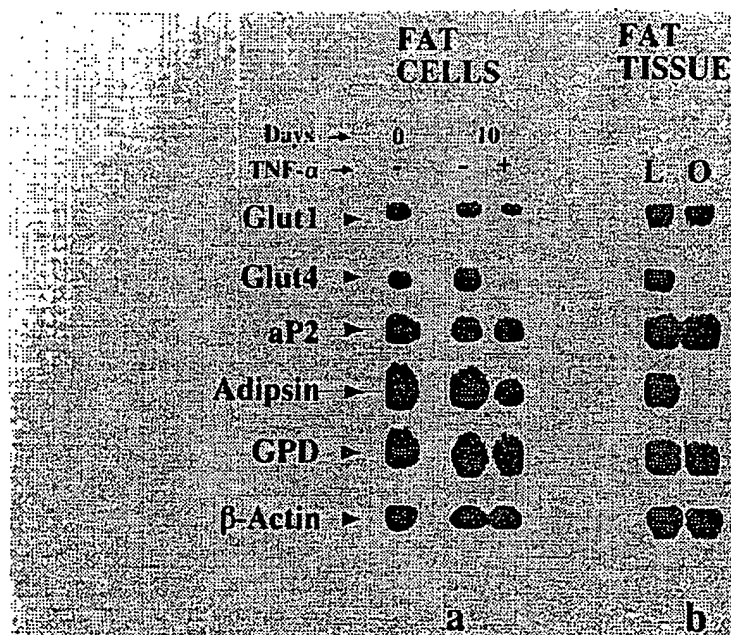
16. The method of claim 1 wherein said therapeutic agent comprises an agent capable of suppressing production of TNF- $\alpha$  or of TNF- $\alpha$  mRNA.

20 17. The method of claim 16 wherein said therapeutic agent comprises methylxanthine.

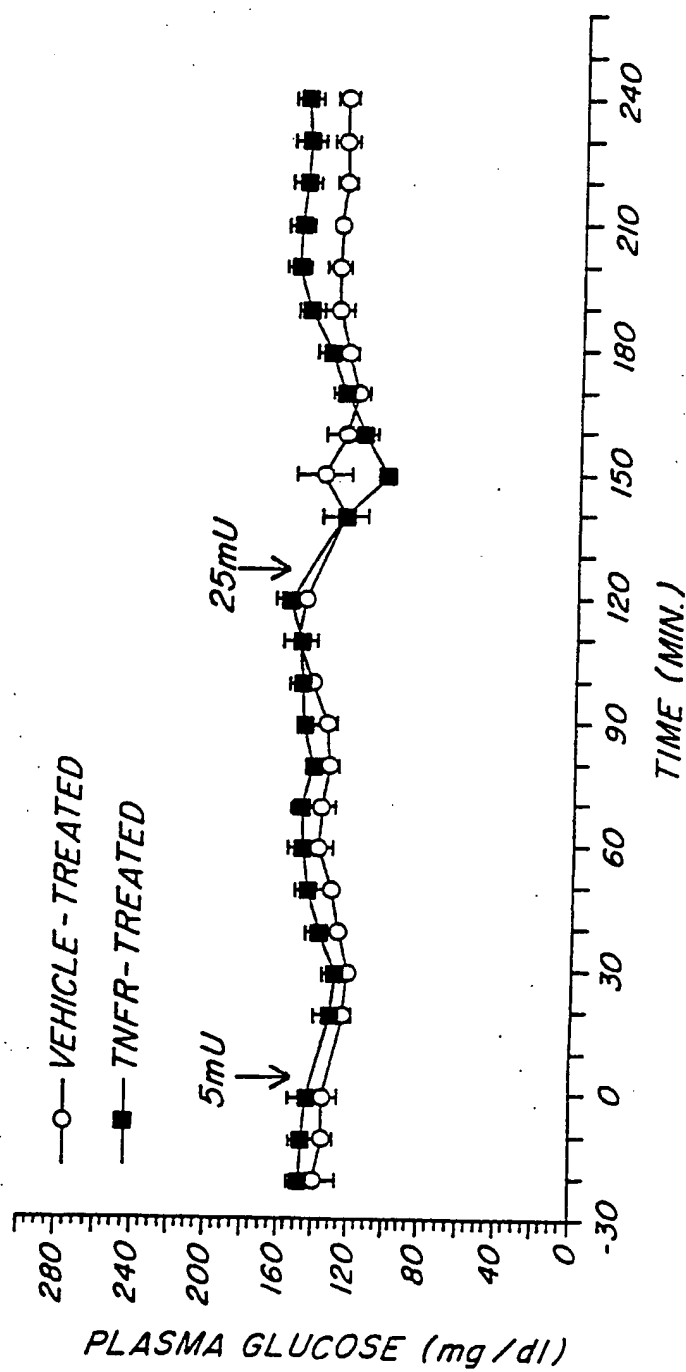
18. The method of claim 16 wherein said therapeutic agent comprises pentoxifylline.

*FIG. 1**FIG. 2*

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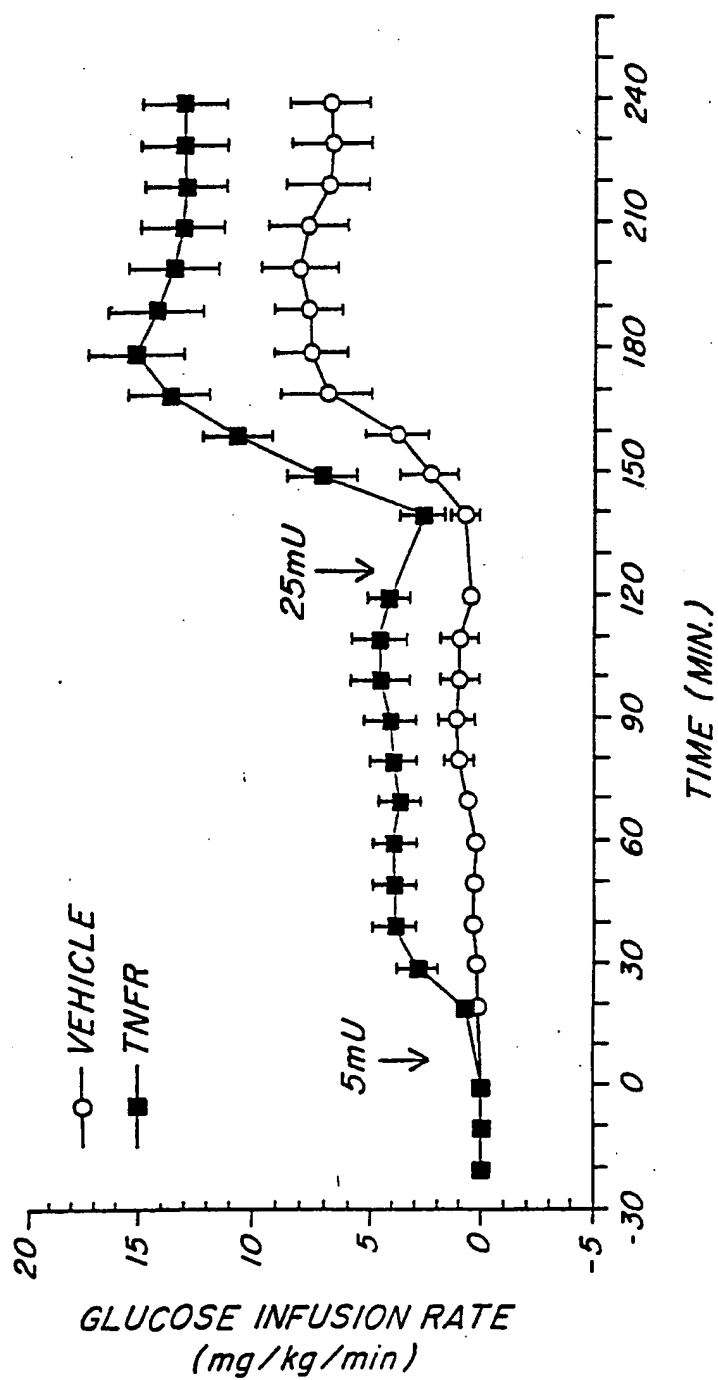
*FIG. 3**FIG. 4*

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**FIG. 5A**



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**FIG. 5B**

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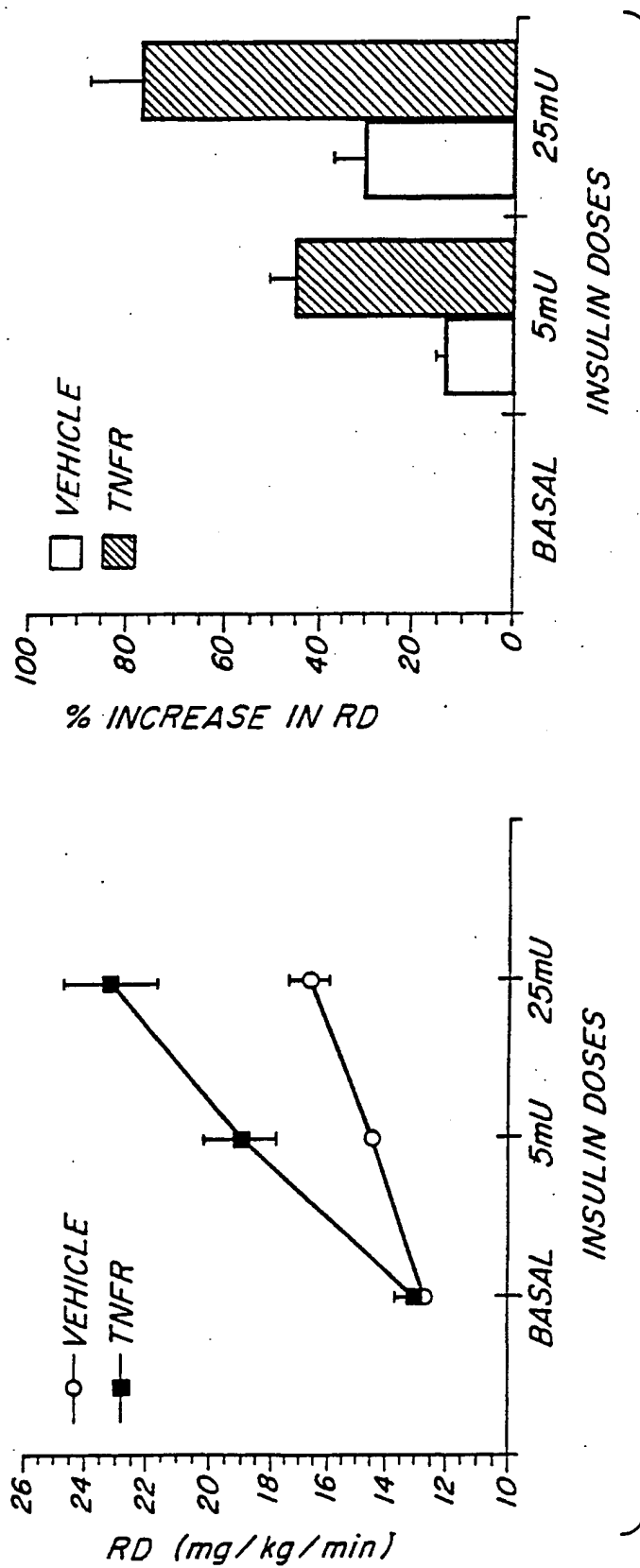
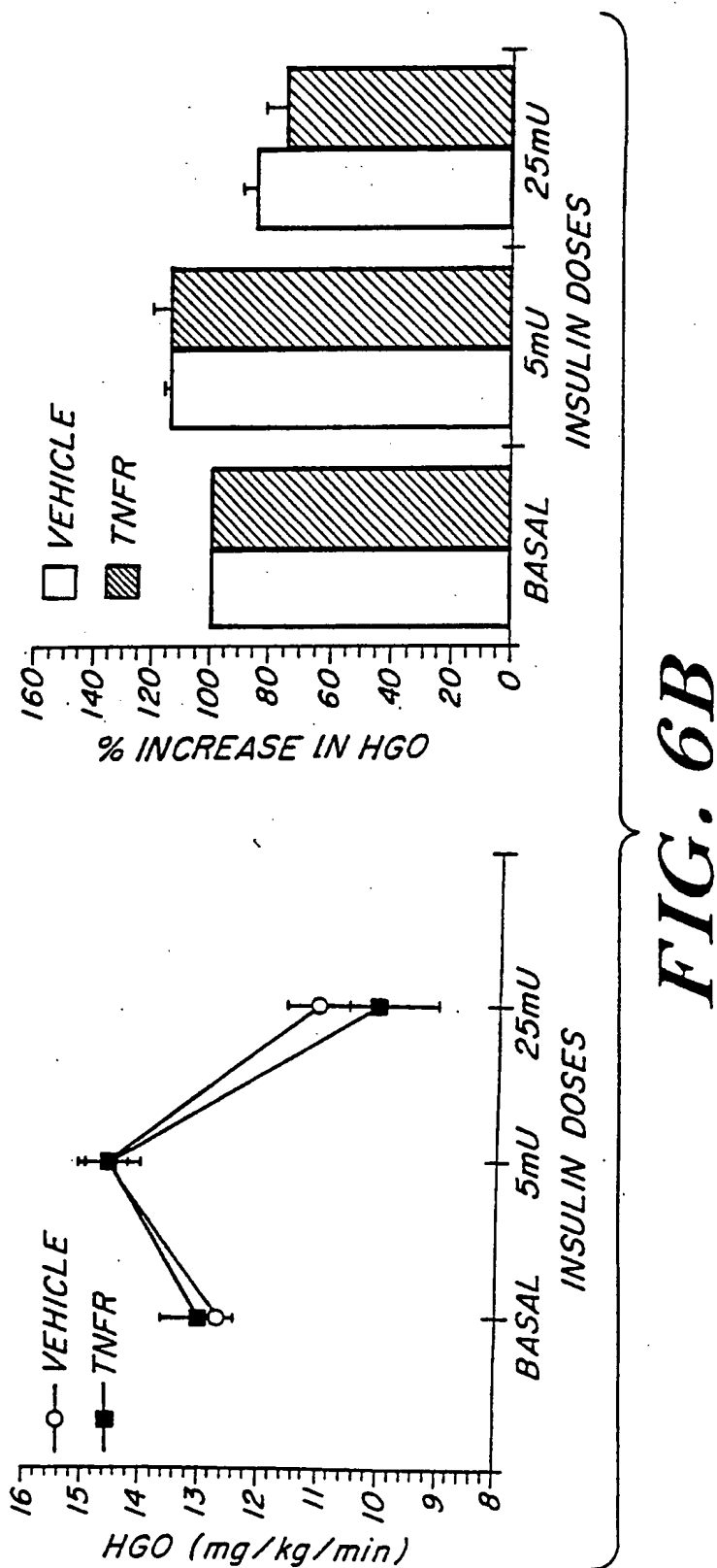


FIG. 6A

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# INTERNATIONAL SEARCH REPORT

Internat. J. Application No  
PCT/US 93/09830

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 5 A61K37/02 A61K39/395 A61K31/52

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 5 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CANADIAN MEDICAL ASSOCIATION JOURNAL vol. 145, no. 12, 15 December 1991, OTTAWA, CANADA pages 1571 - 1581 W. RODGER 'Non-insulin-dependent (type II) diabetes mellitus.' see page 1578, left column, line 41 - line 44	1,16,18
X	ARCHIVES OF INTERNAL MEDICINE vol. 150, no. 4, April 1990, CHICAGO IL, USA page 921 J. AMBRUS ET AL. 'Effect of pentoxifylline on carbohydrate metabolism in type II diabetics (letter).' see page 921, middle column - right column --- -/--	1,16,18

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
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- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

10 January 1994

Date of mailing of the international search report

14. 02. 94

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# INTERNATIONAL SEARCH REPORT

Internat. Application No

PCT/US 93/09830

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Name of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,91 00730 (DAK LABORATORIET A/S) 24 January 1991 see page 6, line 13 - page 20, line 29 ---	1,16,17
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA vol. 88, no. 23 , 1 December 1991 , WASHINGTON DC, USA pages 10535 - 10539 A. ASHKENAZI ET AL. 'Protection against endotoxic shock by a tumor necrosis factor receptor immunoadhesin.' cited in the application see the whole document ---	1-13
P,X	SCIENCE vol. 259, no. 5091 , 1 January 1993 , WASHINGTON DC, USA pages 87 - 91 G. HOTAMISLIGIL ET AL. 'Adipose expression of tumor necrosis factor-alpha: Direct role in obesity-linked insulin resistance.' see the whole document ---	1-3,9-13
P,X	THE JOURNAL OF BIOLOGICAL CHEMISTRY vol. 268, no. 10 , 5 April 1993 , BALTIMORE MD, USA pages 6823 - 6826 B. SPIEGELMAN ET AL. 'Regulation of adipocyte gene expression in differentiation and syndromes of obesity/diabetes.' see page 6825, right column see figure 1 ---	1-3,9-13
P,X	CELL vol. 73, no. 4 , 21 May 1993 , CAMBRIDGE MA, USA pages 625 - 627 B. SPIEGELMAN ET AL. 'Through thick and thin: wasting, obesity, and TNF alpha.' see page 626, left column, line 35 - right column, line 39 -----	1-3,9-13

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat. Application No

PCT/US 93/09830

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		AU-A- 5630890	06-02-91
		CA-A- 2063598	08-01-91
		EP-A- 0480934	22-04-92
		JP-T- 4507091	10-12-92
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